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THE EFFECTS OF OXIDATIVE DAMAGE ON IN VIVO AGING, DENSITY, AND SURVIVAL OF BABOON RED BLOOD CELLS

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To assess the relationships among density, age, and survival of baboon red blood cells, hydrogen peroxide-treated (oxidized) red blood cells and untreated (non-oxidized) red blood cells were labeled with ^{51}Cr and autotransfused. Hydrogen peroxide in a final concentration of 339 umol/liter was used to treat the baboon red blood cells.

Blood samples were collected following autotransfusion of the non-oxidized and oxidized red blood cells. These samples were processed as

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unfractionated red blood cells and as fractionated top, intermediate, and bottom red blood cells consisting of 6 bands isolated using percoll-hypaque density gradients. Oxidized unfractionated red blood cells and oxidized red blood cells in the bottom fraction consisting of 6 bands exhibited significantly reduced ^{51}Cr 24-hour posttransfusion survival values. The T-1/2 values in the bottom fraction were significantly lower in the oxidized than in the non-oxidized red blood cells. As the more dense red blood cells were removed from the circulation, the movement of radioactivity from the less dense top red blood cell fraction to the more dense bottom fraction was reduced.

In another aspect of this study, non-oxidized and oxidized unfractionated and fractionated red blood cells were labeled with biotin. Percoll hypaque density gradients were used to separate the bottom red blood cell fraction containing 3 bands or 6 bands. Twenty minutes after the autotransfusion of these biotin-labeled non-fractionated and fractionated red blood cells, blood samples were collected. The biotin-labeled red blood cells were recovered on avidin plates, labeled with ^{51}Cr, and autotransfused. Samples were collected for 24-hour posttransfusion survival and lifespan measurement.

The 24-hour posttransfusion survival values of the oxidized non-fractionated red blood cells were only 77% compared to 89% for the non-oxidized non-fractionated red blood cells. The oxidized bottom red blood cell fraction containing bands 1, 2, and 3 exhibited a significantly lower 24-hour posttransfusion survival value of 42% than the 89% survival value for the non-oxidized bottom red blood cell fraction containing bands 1 through 6. Red blood cells in band 1 appeared to be more susceptible to oxidation than those in bands 2 through 6 of the bottom red blood cell fraction.

A correlation was observed between red blood cell aging in vivo and an increase in red cell density. Dense red blood cells were more susceptible to in vitro treatment with hydrogen peroxide than were light red blood cells. These irreversibly damaged non-viable dense red blood cells were removed during the 24-hour posttransfusion period and the ^{51}Cr red blood cells in the circulation did not exhibit a reduction in lifespan.

ABSTRACT

To assess the relationships among density, age, and survival of baboon red blood cells, hydrogen peroxide-treated (oxidized) red blood cells and untreated (non-oxidized) red blood cells were labeled with 51Cr and autotransfused. Hydrogen peroxide in a final concentration of 339 umol/liter was used to treat the baboon red blood cells.

Blood samples were collected following autotransfusion of the non-oxidized and oxidized red blood cells. These samples were processed as unfractionated red blood cells and as fractionated top, intermediate, and bottom red blood cells consisting of 6 bands isolated using percoll-hypaque density gradients. Oxidized unfractionated red blood cells and oxidized red blood cells in the bottom fraction consisting of 6 bands exhibited significantly reduced 51Cr 24-hour posttransfusion survival values. The T1/2 values in the bottom fraction were significantly lower in the oxidized than in the non-oxidized red blood cells. As the more dense red blood cells were removed from the circulation, the movement of radioactivity from the less dense top red blood cell fraction to the more dense bottom fraction was reduced.

In another aspect of this study, non-oxidized and oxidized unfractionated and fractionated red blood cells were labeled with biotin. Percoll hypaque density gradients were used to separate the bottom red blood cell fraction

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The 24-hour posttransfusion survival values of the oxidized non-fractionated red blood cells were only 77% compared to 89% for the non-oxidized non-fractionated red blood cells. The oxidized bottom red blood cell fraction containing bands 1, 2, and 3 exhibited a significantly lower 24-hour posttransfusion survival value of 42% than the 89% survival value for the non-oxidized bottom red blood cells fraction containing bands 1 through 6. Red blood cells in band 1 appeared to be more susceptible to oxidation than those in bands 2 through 6 of the bottom red blood cell fraction.

A correlation was observed between red blood cell aging in vivo and an increase in red cell density. Dense red blood cells were more susceptible to in vitro treatment with hydrogen peroxide than were light red blood cells. These irreversibly damaged non-viable dense red blood cells were removed during the 24-hour posttransfusion period and the 51Cr red blood cells in the circulation did not exhibit a reduction in lifespan.

INTRODUCTION

Most studies of red blood cell senescence have utilized a labeled cohort red cell population to identify specific markers of aging.[1-7] Density separation of red blood cells is a method commonly used to isolate the aged red blood cell population.[4,8] Early studies in humans in which 59Fe was used to label a cohort of red blood cells in vivo demonstrated that low density cells become more dense with time,[1,5-8] and it has been suggested that sampling of such cohorts over time would help to identify senescent red blood cells.[9-11] Studies using 59Fe have yielded inconsistent results, presumably because the 59Fe released from senescent red blood cells is reutilized.[9,12,13] As red blood cells within a 59Fe cohort population were destroyed, newly produced red blood cells were randomly labeled in vivo. Recent studies in hypertransfused mice failed to show any significant change in density during red cell aging.[9,11]

In our previous study in baboons, 24-hour posttransfusion survival and T 1/2 values were similar in the non-oxidized less dense 10% of the red blood cells with a MCHC of less than 32 g/dl and the more dense 10% of the red cells with a MCHC of 37 g/dl.[10] However, assessments of the alterations in membrane structure in vitro and of 24-hour posttransfusion survival values showed that the bottom 10% of the red blood cells were significantly more sensitive to oxidant damage than the top 10%.[10]

In our study reported here, the age, density and survival of non-oxidized and oxidized unfractionated and fractionated baboon red blood cells were assessed. The oxidized red blood cells were prepared using 339 umol/L hydrogen peroxide. The non-oxidized and oxidized red blood cells were labeled with 51Cr and autotransfused, following which blood samples were collected. Using percoll-hypaque gradients, some of the red blood cells were separated into the top red blood cell fraction, intermediate red blood cell fraction, and bottom red blood cell fraction containing 6 bands. Twenty-four hour posttransfusion survival and lifespan were measured in these red blood cell fractions as well as in unfractionated red blood cells.

We also studied non-fractionated and fractionated bottom red blood cells containing 3 bands and 6 bands. These red blood cells were labeled with biotin and autotransfused. Twenty minutes after the autotransfusion, the biotin-labeled red blood cells were recovered on avidin plates, labeled with 51Cr, and autotransfused. Blood samples were collected, and measurements were made of 24-hour posttransfusion survival and T 1/2 values.

MATERIALS AND METHODS

Preparation of hydrogen peroxide treated red blood cells. A 40 ml volume of venous blood was collected from each baboon into 5.5 ml of the anticoagulant citrate phosphate dextrose (CPD). The blood was centrifuged to remove the plasma and leukocytes, and the red blood cells were washed once with phosphate buffered saline (PBS: 5 mmol/L phosphate, 0.15 mol/L NaCl, and 5 mmol/L dextrose, pH 7.4) and resuspended to a final hematocrit of 20% in PBS with 2 mmol/L sodium azide to inhibit catalase. The red blood cells were incubated at 30 C for 10 minutes, the hydrogen peroxide was added to achieve a final concentration of 339 umol/L, and the red blood cells were incubated for an additional 10 minutes at 30 C. The red blood cells were washed with phosphate buffered saline (PBS) containing 5 mmol/L dithiothreitol (DTE) and then incubated for 5 minutes in the same buffer to stop the oxidation process. Other red blood cells (non-oxidized) were treated in a similar manner but were not treated with hydrogen peroxide.

Separation of red cells on density gradients. Red blood cell fractions of specific densities were isolated using percoll hypaque density gradients.[14] A suspension of non-oxidized or oxidized washed baboon red blood cells was mixed thoroughly with a mixture of percoll and hypaque (40% percoll, 40% distilled water, 20% hypaque; 10 cm red blood cells/160 cm percoll-hypaque mixture in a Sorvall Dupont 18

mm X 100 mm polycarbonate tube 16 ml nominal capacity) and were centrifuged for 20 minutes at 15,000 (ss-24 rotor, Sorvall). The uppermost layer of cells on the gradient, containing predominantly white cells and reticulocytes, was aspirated and discarded. The top 10% and bottom 5% of the red blood cells in the gradient were carefully isolated and washed three times in phosphate buffered saline. Mean corpuscular hemoglobin concentration (MCHC) and pyrimidine 5′ nucleotidase were measured in each fraction to determine the reproducibility of the separation technique.[15,16,17] Hemoglobin recovery for each fraction was determined from measurements of hemoglobin concentration and volume of the sample prior to fractionation and on each of the separate fractions.

In Vitro 51Cr distribution studies. Studies were done to determine the effect of in vitro oxidation of red blood cells on the distribution of 51Cr in the red blood cell fractions. Whole blood was collected into the CPD anticoagulant and the red blood cells were isolated: the red blood cells were washed with PBS and labeled with 51Cr. The 51Cr-labeled cells were then divided into two equal volumes: one volume was treated with 339 umol/L hydrogen peroxide to produce oxidation; the other volume was not treated and served as the control. The red blood cells were washed with PBS, incubated at 37 C for 20 minutes, and then separated on percoll hypaque density gradients. The 51Cr

radioactivity and hemoglobin recovery was measured in each layer.

In vivo RBC survival measurements. In vivo measurements were made under sterile conditions. The solutions used during the procedure were sterilized either by steam autoclaving or by filtering through a 0.2 umol/L filter (MSI, Westboro, MA). Penicillin G (2500 U/mL) was added to the PBS buffer used to wash the red blood cells. The sterility of the red blood cell suspension was determined by microbiologic testing using blood agar plates to detect aerobic organisms and thioglycollate broth tubes to detect aerobic and anaerobic organisms.

Red blood cell survival was quantitated using the double Cr 51 labeling method previously described.[18,19] A 50-60 ml volume of red blood cells was labeled with Cr 51 and infused into the baboon. Measurements of 24-hour posttransfusion survival and the T 1/2 values were made in blood samples taken prior to and 5 and 20 minutes and 1, 7, 14, and 21 days following the transfusion of the non-oxidized or oxidized autologous red blood cells.

Post-infusion samples were collected and studied as unfractionated and fractionated red blood cells. Percoll-hypaque density gradients were used to isolate the top, intermediate and bottom fractions of the red blood cells. The top fraction containing 10% of the total hemoglobin and the bottom fraction consisting of bands 1 through 6 and

containing 5% of the total hemoglobin in the gradient were carefully isolated and washed three times in PBS. The total hemoglobin value of the unfractionated sample was determined by measuring the volume of the sample and the hemoglobin concentration.

Twenty-four-hour postinfusion survival (%) and lifespan (T50, days) values were calculated in the unfractionated red blood cells and in the top and bottom red blood cell fractions by comparing the Cr 51 counts per minute per gram of hemoglobin (cpm/g hb) in the 5-minute postinfusion samples to the cpm/g hb in the subsequent post-infusion samples. Means $\pm$ 1 SD were calculated for each experimental group, and comparisons between groups were made using the Student's *t*-test.

Preparation of non-oxidized and oxidized, biotin-labeled baboon red blood cells. One hundred twenty milliliters of blood were collected from each male baboon into 16 ml citrate phosphate dextrose anticoagulant.

1) Oxidized Unfractionated Red Blood Cells: Red blood cells were treated with hydrogen peroxide (339 umol/L) as described above and treated with biotin.

2) Oxidized Bottom Fraction Red Blood Cells: Following oxidation with hydrogen peroxide, the red blood cells were separated on percoll hypaque density gradients, and the bottom fraction containing bands 1, 2 and 3 was isolated and treated with biotin.

3) Non-oxidized Unfractionated Red Blood Cells: The red blood cells were washed and then treated with biotin.

4) Non-oxidized, Bottom Fraction Red Blood Cells: The red blood cells were washed, and then isolated on percoll hypaque density gradients. The bottom fraction containing bands 1 through 6 was isolated and biotin-treated.

Because the red blood cells in the bottom fraction redistributed following oxidation, it was necessary to isolate bands 1-6 of the bottom fraction of the non-oxidized sample to get an equivalent number of red blood cells as bands 1-3 of the bottom fraction of the oxidized red blood cells.

All red blood cells were resuspended to a 10% final hematocrit in phosphate buffered saline containing 15 mmol/L glucose prior to treatment with biotin.

Biotin Treatment: The oxidized and non-oxidized unfractionated red blood cells and the oxidized and non-oxidized bottom fractions of the red blood cells were treated with biotin by the procedure reported by Dale and Norenberg.[20] Sucinnylated BSA (295 nmol) was added to the cell suspension, mixed thoroughly and incubated for 3 minutes at 37 C.[10,17,20] This was followed by addition of NHS-biotin (295 nmol) and incubation at 37 C for an additional 60 minutes. The biotin-treated red blood cells were washed three times with PBS, resuspended to a final hematocrit of 30% in PBS containing 1 mg/ml BSA and 5 mmol/L

glucose, and autotransfused. Twenty minutes following autotransfusion, a 300 ml volume of blood was withdrawn and the biotin-treated red blood cells were recovered on avidin plates (15 cm polystyrene petri dishes, Scientific Products, McGraw Park, IL).

The plates were prepared as follows: 1.8 mg biotin-treated gelatin in 5 ml PBS was added to each plate;[10,17,20] the plate was washed several times with PBS; 0.4 mg avidin in 5 ml PBS was added and the plate was rocked for 60 minutes and then washed with PBS. Three milliliters of the red cell suspension were added to the plate, the plate was gently rocked for 60 minutes, and the unbound biotin-treated red blood cells were gently rinsed from the dish with a transfer pipette and autotransfused.

The bound red blood cells were removed from the plate by adding 5 ml of PBS containing 0.2 mg/ml collagenase (type IV, 445 U/ml), 1 mg/ml BSA and 0.1 mmol/L biotin and rocking for 15 minutes. In some instances, it was necessary to gently agitate the red blood cells with a transfer pipette to release them from the plate. The biotin-treated and avidin-isolated red blood cells were washed three times in glucose phosphate buffered saline and labeled with 51Cr.

Survival of Biotin-Treated Avidin-Isolated Red Blood Cells: About 100 ml of red blood cells that did not bind to the avidin platelets were recovered and autotransfused to restore the baboon's blood volume. Thirty minutes following

the autotransfusion, the baboon's red blood cell volume was estimated from the 125I-albumin measured plasma volume and the total body hematocrit (peripheral venous hematocrit multiplied by 0.87).[19]

A 3 to 5 ml volume of biotin-treated and avidin-isolated 51Cr labeled red blood cells with a hematocrit of 1 to 4 V% was autotransfused. Samples were obtained at 5, 10, 15, 20, and 30 minutes and 1, 2, 7, 14, and 21 days following infusion to determine the 24-hour survival (%) and lifespan (T50, days) of the biotin-treated and avidin-isolated 51Cr-labeled red blood cells.

24-Hour Posttransfusion Survival and Lifespan of Small Volumes of Autologous Fresh Baboon Red Blood Cells. Three studies were done to determine the accuracy of the 24-hour posttransfusion survival and lifespan measurements in small volumes of fresh autologous baboon red blood cells similar to the volume of the transfused biotin-treated and avidin-isolated 51Cr-labeled red blood cells. A 10 ml volume of baboon blood was collected into 1.5 ml of citrate phosphate dextrose anticoagulant. The red blood cells were washed with 0.9% sodium chloride, labeled with 51 Cr and diluted with 0.9% sodium chloride. The hematocrit was adjusted to a value of 2 V%: the value was estimated from the red blood cell count and mean corpuscular volume (MCV) measured in the Coulter Counter Model JT, Edison, NJ. A 3 ml volume of washed red blood cells with a hematocrit of 1 to 1.5 V% was

labeled with 51Cr and autotransfused. Samples were drawn prior to and 5, 10, 15, 20, and 30 minutes and 1, 2, 7, 14, and 21 days after the autotransfusion for 24-hour posttransfusion survival and lifespan measurements. The 24-hour posttransfusion survival was determined by comparing the 51Cr counts per minute per ml of red blood cell in the 5-minute post-infusion samples to counts in the subsequent post-infusion samples.

RESULTS

Density Gradient Separation: The percoll-hypaque density gradients separated the baboon red blood cells into three fractions: the top and intermediate, and the bottom fraction consisting of 6 bands. There was a change in thickness in bands 1 and 2 (the upper bands) of the bottom fraction following oxidation, and prior to and 20 minutes after transfusion, and 24-hours after transfusion. Band 1 showed a reduction in thickness 20 minutes after transfusion and a further reduction 24-hours after transfusion as the thickness of band 2 increased. These changes suggested either that the red blood cells were being removed from the circulation or that the density of some of the red blood cells increased in band 1 and these dense red blood cells were transferred to band 2. These findings also suggest that oxidant injured red blood cells were present in band 1 of the 6 bands in the bottom fraction of red blood cells.

The density gradient separations were reproducible as assessed by the measurements of mean corpuscular hemoglobin concentration (MCHC) and pyrimidine 5′ nucleotidase levels, as well as the percentage of hemoglobin in each fraction (Table 1). The top less dense red blood cell fractions contained 10% of the total hemoglobin, and the bottom more dense red blood cell fractions contained 5% of the total hemoglobin. Red cell density correlated positively with MCHC for both non-oxidized and oxidized fractions[21]: denser red blood cells had significantly higher MCHC values.

The pyrimidine 5′ nucleotidase level correlated inversely with red blood cell density ($P<0.01$)[15,16]: denser red blood cells had significantly lower levels of pyrimidine 5′ nucleotidase. The low variability observed in MCHC and pyrimidine 5′ nucleotidase levels demonstrated the effectiveness of the percoll hypaque density gradient procedure in isolating the less dense 10% of the red blood cells and the more dense 5% of the red blood cells.

Determination of 51Cr Radioactivity in Top, Intermediate, and Bottom Fractions of the Red Blood Cells Following Transfusion: Non-oxidized and oxidized 51Cr-labeled baboon red blood cells were infused. Five and 20 minutes, 24-hours, and 7, 14, and 21 days following transfusion, blood samples were collected and fractionated on percoll-hypaque density gradients.

Figure 2 reports the distribution of 51Cr (reported as counts per minute per gram of hemoglobin) in the non-oxidized top, intermediate, and bottom red blood cell fractions. The 51Cr radioactivity in the top red blood cell fractions decreased progressively over the 24-hour to 21-day posttransfusion period. The loss of 51Cr was slower in the bottom red blood cell fractions. The difference ($p<0.05$) in 51Cr radioactivity between the top and bottom red blood cell fractions was significant during the 14- to 21-day post-infusion period. To determine whether there was differential elution of 51Cr from red blood cells in the

top, intermediate, and bottom fractions, the top and bottom red blood cell fractions were incubated at 37 C for 24-hours, and 51Cr elution patterns were analyzed in vitro. The unfractionated red blood cells retained 94% ± 1.5, the top fraction retained 92.0% ± 3.0, and the bottom fraction retained 95% ± 1.0 of their original 51Cr radioactivity, indicating that there was no preferential elution of 51Cr from the top and bottom cells during the in vitro incubation.

Figure 3 reports the effects of hydrogen peroxide treatment on the in vivo 51Cr distribution in the top, intermediate and bottom fractions of the baboon red blood cells. The bottom fraction of the hydrogen peroxide-treated red cells exhibited a significant reduction in 51Cr radioactivity during the 24-hour to 7-day posttransfusion period ($P<0.05$), but neither the top nor the bottom fraction showed any significant difference during the 14- to 21-day posttransfusion period.

24-hour Posttransfusion Survival and T1/2 Values in The Top and Bottom Fractions of Non-Oxidized and Oxidized Red Blood Cells: Table 2 reports the 24-hour posttransfusion survival and T 1/2 of non-oxidized and oxidized unfractionated and fractionated top and bottom red blood cells. The 24-hour posttransfusion survival value of 81% for the oxidized unfractionated red blood cells was significantly lower than the 95% value for the non-oxidized unfractionated red blood

cells, but the T 1\2 values were similar. The top and bottom fractions of the non-oxidized red blood cells had similar 24-hour post-transfusion survival values of 95% and 91% respectively. However, T 1/2 values were significantly ($p<0.05$) different in the two groups: 9.8 $\pm$ 1.4 days for non-oxidized top fraction and 21 $\pm$ 4.3 days for the non-oxidized bottom fraction. These results indicate that following transfusion the non-oxidized 51Cr labeled red cells moved from the top less dense fractions to the bottom more dense fractions, resulting in a much longer T 1\2 value in the bottom fraction. The oxidized red blood cells, on the other hand, exhibited no such phenomenon. The bottom fraction of the oxidized red blood cells exhibited a 13% lower 24-hour posttransfusion survival than the top fraction, and the T 1/2 of these red blood cells was 13.3 $\pm$ 0.7 days. The irreversibly damaged oxidized red blood cells were removed from the circulation during the 24-hour posttransfusion period.

Effect of Oxidation on the Distribution of the 51Cr-Labeled Red Blood Cells in the Top, Intermediate, and Bottom Fractions: The red blood cell unit was labeled with 51Cr and separated into two parts. One sample was oxidized with 339 umol/L of hydrogen peroxide and the other was not. Each sample was fractionated on percoll hypaque density gradients into top, intermediate, and bottom fractions. The percent of total radioactivity and the recovery of hemoglobin were

calculated for each fraction. No statistically significant differences were observed in the 51Cr radioactivity in the top, intermediate, and bottom layers of the non-oxidized and oxidized baboon red blood cells (Table 3).

Autotransfusion of Non-Oxidized and Oxidized Unfractionated and Fractionated Biotin-Labeled Bottom Red Blood Cells Containing 1 to 3 or 1 to 6 Bands: Autotransfused Biotin-Labeled Red Blood Cells were Recovered on Avidin Plates, Labeled with 51Cr, and Autotransfused for Measurement of 24-Hour Posttransfusion Survival and T 1\2 Values: Normal 24-hour posttransfusion survival values were observed for both non-oxidized unfractionated red blood cells (88.7%) and non-oxidized bottom fractions containing 1 through 6 bands (89%). _Oxidized unfractionated red blood cells had significantly reduced 24-hour posttransfusion survival values (76.7% ± 1.70), and oxidized bottom red blood cell fractions containing 1, 2, and 3 bands had reduced 24-hour posttransfusion values of 41.8% ± 19.6 (p 0.05).

Lifespan values were within normal limits for both non-oxidized and oxidized unfractionated red blood cells and non-oxidized and oxidized bottom red blood cell fractions (Table 4).

Table 5 reports the 24-hour posttransfusion survival and T 1/2 values in 3 healthy baboons autotransfused with a 3 ml volume of washed red blood cells with a hematocrit of 1 to 1.5 V%. The hematocrit was estimated from the red blood

cell count and the mean cell volume (MCV) measured in the Coulter Counter. The finding of normal 24-hour posttransfusion survival (91%) and the T 1/2 (14.7 days) values in samples indicate that accurate measurements of 24-hour posttransfusion survival and lifespan values can be made in small volume samples.

Bacteriologic cultures were negative in the non-oxidized and oxidized unfractionated red blood cells and bottom red blood cell fractions following labeling with biotin, autotransfusion, recovery on avidin plates, labeling with 51Cr and subsequent autotransfusion.

DISCUSSION

In a previous study from our laboratory, baboons were autotransfused with oxidized and non-oxidized red blood cells separated by percoll hypaque density gradients and labeled with 51Cr. The 24-hour posttransfusion survival and the T 1/2 values in the non-oxidized were similar in the top less dense 10% and the bottom more dense 10% of the red blood cells. (10) The oxidized red blood cell bottom fraction exhibited significantly lower 24-hour posttransfusion survivals (70%) than the non-oxidized bottom red blood cell fraction (89%), although the T 1/2 values of the non-oxidized and oxidized bottom red blood cell fractions were not significantly different.(10)

In the study reported here, non-oxidized and oxidized unfractionated baboon red blood cells were labeled with 51Cr and autotransfused. Following the autotransfusion, blood samples were collected and separated into two portions. One portion was treated with percoll hypaque density gradients to separate the top fraction, intermediate fraction, and the bottom fraction containing 6 bands; the other portion was not treated with percoll hypaque gradients. Twenty-four hour post-transfusion and T 1/2 values in the non-oxidized red blood cells demonstrated a significant correlation between red blood cell age and density.

Non-oxidized 51Cr-labeled red blood cells were shown to become more dense as they circulated <u>in vivo</u>, and 51Cr

radioactivity was found to accumulate in the bottom more dense red blood cell fractions. The T 1/2 value was 21 days for the non-oxidized bottom red blood cells compared to 12.3 days for the non-oxidized unfractionated red blood cells and 9.8 days for non-oxidized top red blood cell fractions (Figure 2 and Table 2). The oxidized red blood cells exhibited a reduced thickness of band 1 of the bottom red blood cell fraction 20 minutes after transfusion compared to that prior to transfusion, and a further reduction 24 hours after transfusion. Two explanations for the posttransfusion decrease in band 1 and increase in band 2 of the bottom red blood cell fraction were considered: either that the red blood cells were removed from the circulation, or that the density of some of the red blood cells in band 1 increased and the more dense red blood cells moved to band 2. These data suggest that oxidation with 339 umol/L of hydrogen peroxide irreversibly damaged the least dense band 1 of the bottom fraction of the red blood cells and that these damaged red blood cells were removed during the 24-hour posttransfusion period without a reduction in the lifespan. The rapid removal of the oxidized red blood cell fraction accounted for the lack of accumulation of 51Cr radioactivity in the circulating more dense oxidized red blood cells (Figures 2 and 3).

The 51Cr 24-hour posttransfusion survival of the top red blood cell fraction was not significantly different

between non-oxidized and oxidized red blood cells (Figures 2 and 3). The reduced radioactivity in the top, intermediate and bottom red blood cell fractions may have been the result of dilution of these cells with the newly circulating nonradioactive reticulocytes, the movement of 51Cr-labeled red blood cells from the less dense to the more dense layers following transfusion, and/or the removal of the 51Cr-labeled red blood cells from the circulation. The lifespan value was 12.3 days for the non-oxidized unfractionated red blood cells, 9.8 days for the non-oxidized top fraction of the red blood cells, and 21 days for the non-oxidized bottom fraction of the red blood cells (Table 2). The red blood cells in the oxidized bottom fraction were rapidly removed from the circulation, with a 24-hour posttransfusion survival of 78% and a T 1/2 value of 13.3 days. Our data demonstrated a relation between red blood cell age and density.

A complex relation exists between red blood cell density and survival.[21-23]

Oxidized red blood cells in the bottom fraction containing 1 to 6 bands had a 51Cr 24-hour posttransfusion survival value of 78%, whereas non-oxidized red blood cells in this fraction had a survival value of 91%. Following biotin labeling and autotransfusion, recovery on avidin plates 20 minutes later, and 51Cr labeling and

autotransfusion, the 24-hour posttransfusion survival value was 42% for the oxidized red blood cells in the bottom fraction containing 1, 2, and 3 bands compared to 89% for the non-oxidized red blood cells in the bottom fraction containing bands 1 through 6. This finding suggests that hydrogen peroxide treatment produced more damage to bands 1,2 and 3 than to bands 1 through 6 in the bottom red blood cell fraction. The changes in bands 1 and 2 in the bottom fraction of the oxidized red blood cells prior to and 20 minutes and 24-hours after transfusion suggest that the oxidized red blood cells in band 1 were more susceptible to oxidative damage than the red blood cells in bands 2 through 6 of the bottom fraction. The data also show that the 24-hour posttransfusion survival value and not the lifespan detected the irreversibly damaged dense red blood cells.

TABLE 1

MEAN CORPUSCULAR HEMOGLOBIN CONCENTRATION (MCHC) AND PYRIMIDINE 5′ NUCLEOTIDASE LEVEL IN NON-TREATED AND HYDROGEN PEROXIDE TREATED UNFRACTIONATED AND TOP, INTERMEDIATE, AND BOTTOM FRACTIONS ISOLATED BY PERCOLL HYPAQUE DENSITY GRADIENTS

SAMPLE	MCHC (gms/dl)		PYRIMIDINE 5′ NUCLEOTIDASE (nmoles/min/mg Hb)		% OF CELLS IN EACH LAYER
CONTROL--NOT TREATED (Mean + SD)					
Unfractionated	32.7 ± 0.9	n=26	0.65 ± 0.07	n=3	---
Top	31.3 ± 0.9	n=27	1.28 ± 0.19	n=3	10.2 ± 3.6
Intermediate	33.1 ± 1.1	n=27	0.65 ± 0.06	n=3	---
Bottom (Bands 1-6)	36.8 ± 1.9	n=27	0.27 ± 0.02	n=3	4.8 ± 1.7
OXIDIZED--339 umol/L Hydrogen Peroxide					
Unfractionated	32.9 ± 1.5	n=22	0.54 ± 0.08	n=3	---
Top	31.3 ± 1.7	n=22	0.96 ± 0.14	n=3	10.1 ± 3.4
Intermediate	33.7 ± 1.5	n=22	0.68 ± 0.10	n=3	---
Bottom (Bands 1-6)	37.5 ± 2.6	n=27	0.26 ± 0.02	n=3	5.3 ± 2.4

TABLE 2

BABOON BLOOD SAMPLES WITH AND WITHOUT 339 umol/L OF HYDROGEN PEROXIDE TREATMENT WERE 51CR LABELED AND AUTOTRANSFUSED. FOLLOWING TRANSFUSION, BLOOD SAMPLES WERE COLLECTED AND PROCESSED AS UNFRACTIONATED RED BLOOD CELLS AND RED BLOOD CELLS SEPARATED USING PERCOLL HYPAQUE DENSITY GRADIENTS INTO TOP, INTERMEDIATE AND BOTTOM FRACTIONS TO ASSESS THE 24-HOUR POSTTRANSFUSION SURVIVAL AND LIFESPAN

	UNFRACTIONATED		TOP FRACTION		BOTTOM FRACTION (BANDS 1-6)	
	24-HOUR SURVIVAL, %	T50 (DAYS)	24-HOUR SURVIVAL, %	T50 (DAYS)	24-HOUR SURVIVAL, %	T50 (DAYS)
CONTROL--NOT TREATED						
MEAN	95	12.3	95	9.8	91	21.0
SD	7	0.7	4	1.4	3	4.3
N	4	4	4	4	3	3
	*				*	*
HYDROGEN PEROXIDE TREATED						
MEAN	81	12.1	90	9.9	78	13.3
SD	2	1.1	12	1.3	2	0.7
N	4	4	4	4	3	3

*P<0.05, Significant difference between control--non-treated and hydrogen peroxide treated red cells.

TABLE 3

EFFECT OF OXIDATION ON THE DISTRIBUTION OF 51CR RED BLOOD CELL RADIOACTIVITY IN THE TOP, INTERMEDIATE, AND BOTTOM FRACTIONS ISOLATED BY PERCOLL-HYPAQUE DENSITY GRADIENTS IN VITRO

MCHC (gm/dl)	FRACTIONS	% OF 51CR RADIOACTIVITY	N
CONTROL SAMPLES--NOT TREATED (Mean + SD)			
31.0 ± 2.1	Top	10.27 ± 4.7	4
32.5 ± 1.6	Intermediate	76.68 ± 4.6	4
35.8 ± 0.5	Bottom (Bands 1-6)	13.05 ± 1.6	4
OXIDIZED SAMPLES--TREATED WITH 339 umol/L OF HYDROGEN PEROXIDE			
30.6 ± 0.9	Top	8.75 ± 3.0	4
32.3 ± 1.5	Intermediate	78.75 ± 1.8	4
34.3 ± 1.0	Bottom (Bands 1-6)	12.50 ± 4.4	4

TABLE 4

BABOON BLOOD WITH AND WITHOUT TREATMENT WITH 339 umol/L OF HYDROGEN PEROXIDE WAS PROCESSED AS UNFRACTIONATED RED BLOOD CELLS AND RED BLOOD CELLS SEPARATED BY PERCOLL HYPAQUE DENSITY GRADIENTS INTO BOTTOM RED BLOOD CELL FRACTION CONTAINING BANDS 1 THROUGH 6 OR ONLY BANDS 1 THROUGH 3, BIOTIN LABELED AND AUTOTRANSFUSED. TWENTY MINUTES AFTER TRANSFUSION, BLOOD WAS COLLECTED AND THE BIOTIN LABELED RED BLOOD CELLS WERE ISOLATED ON AVIDIN PLATES AND 51CR LABELED. THE 24-HOUR POSTTRANSFUSION SURVIVAL AND THE LIFESPAN OF THE 51CR LABELED CONTROL NON OXIDIZED UNFRACTIONATED RED BLOOD CELLS, THE CONTROL NON-OXIDIZED BOTTOM RED BLOOD CELL FRACTION CONTAINING BANDS 1 THROUGH 6; THE OXIDIZED UNFRACTIONATED RED BLOOD CELLS, AND THE OXIDIZED BOTTOM RED BLOOD CELL FRACTION CONTAINING BANDS 1 THROUGH 3 ARE REPORTED.

SAMPLE	N	24-HOUR POSTTRANSFUSION SURVIVAL, %	T50 (DAYS)
CONTROL			
--UNFRACTIONATED RED BLOOD CELLS	3	88.7 ± 4.1	13.0 ± 1.1
--BOTTOM RED BLOOD CELLS (BANDS 1-6)	2	89.0 ± 4.2	14.4 ± 3.4
OXIDIZED			
--UNFRACTIONATED RED BLOOD CELLS	4	76.7 ± 1.7	12.2 ± 1.3
--BOTTOM RED BLOOD CELLS (BANDS 1-3)	4	41.8 ± 19.6	11.2 ± 2.3

TABLE 5

24-HOUR POSTTRANSFUSION SURVIVAL AND LIFESPAN OF A 3 ML VOLUME OF RED BLOOD CELLS WITH A HEMATOCRIT OF 1 TO 1.5 V% AUTOTRANSFUSED INTO THE BABOON

	24-HOUR POSTTRANSFUSION SURVIVAL (%)	LIFESPAN (T50, DAYS)
MEAN	91	14.7
SD	2	0.8
N	3	3

FIGURE LEGENDS

FIGURE 1: Percoll hypaque density separation of: non-oxidized baboon blood (A), hydrogen peroxide treated baboon blood (B), hydrogen peroxide treated baboon blood 20 minutes after transfusion (C), and hydrogen peroxide treated baboon blood 24-hours after transfusion (D). Baboon red blood cells were separated by percoll hypaque density into the top fraction, intermediate fraction, and bottom fraction consisting of 6 bands. Bands 1 and 2 in the upper portion of the bottom fraction changed in thickness following oxidation and prior to transfusion (B), 20 minutes after transfusion (C), and 24-hours after transfusion (D). Band 1 of the bottom fraction showed a decrease in thickness in the 20 minute post-transfusion sample compared to the pre-transfusion sample, and a further decrease 24 hours posttransfusion. Band 2 of the bottom fraction showed an increase in thickness 24-hours after transfusion compared with 20 minutes after infusion.

FIGURE 2: ^{51}Cr counts per minute per gram of hemoglobin (cpm/g hb) in top fraction, intermediate fraction and bottom fraction consisting of 6 bands in the non-oxidized red blood cells following infusion.

FIGURE 3: 51Cr cpm/g hb in top fraction, intermediate fraction and bottom fraction consisting of 6 bands in oxidized red blood cells following infusion.

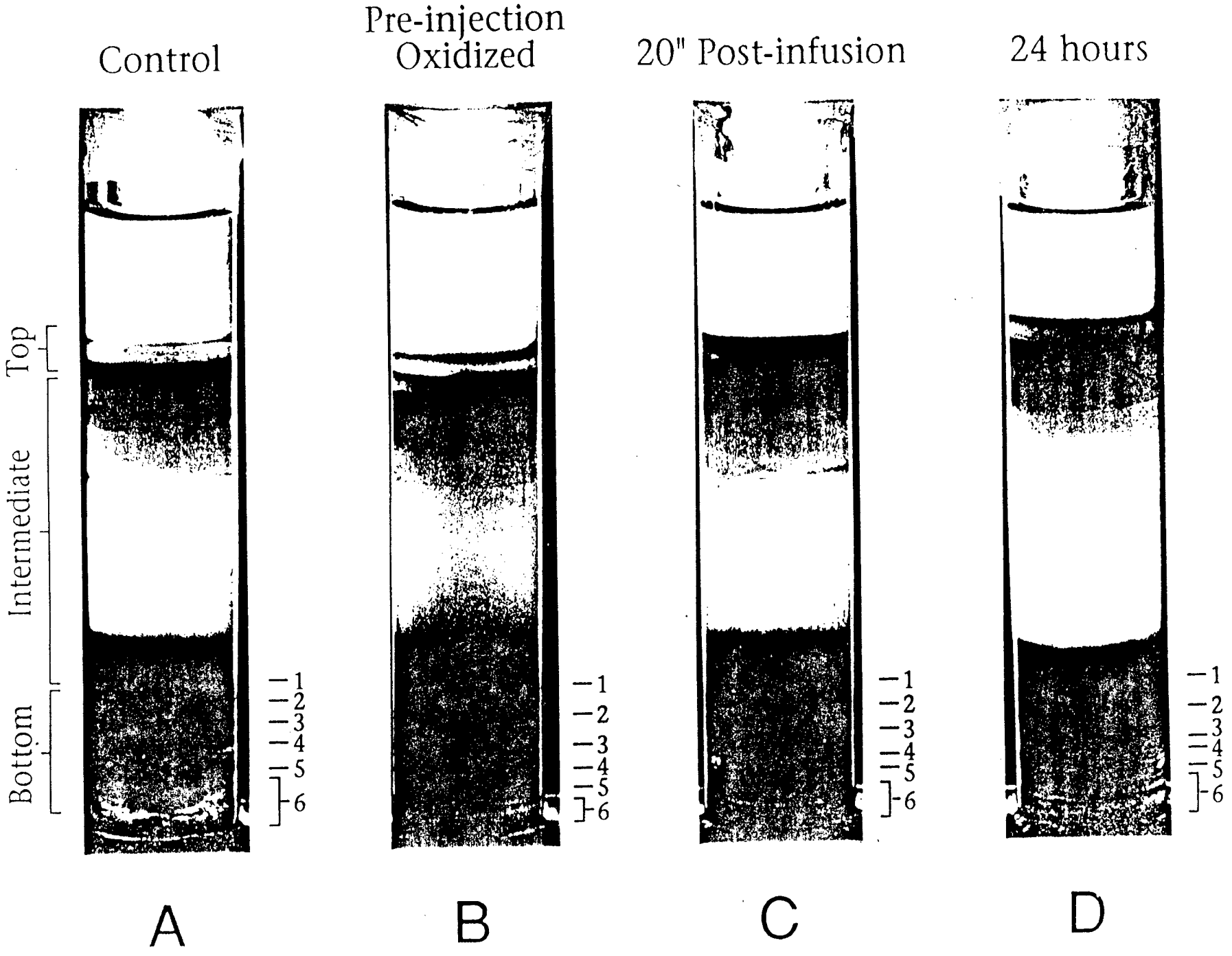
Control
Pre-injection
Oxidized
20" Post-infusion
24 hours
Top
Intermediate
Bottom
1
2
3
4
5
6
A
B
C
D

Figure 1

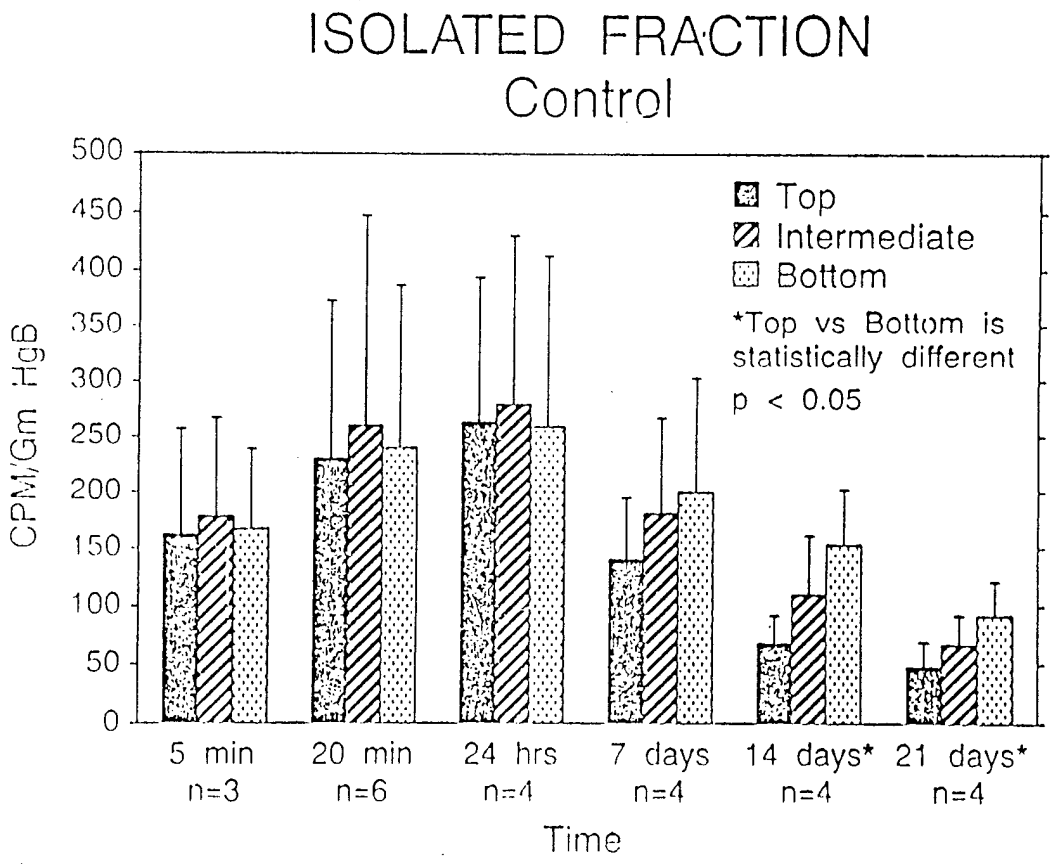
ISOLATED FRACTION
Control
CPM/Gm HgB
500
450
400
350
300
250
200
150
100
50
0
Top
Intermediate
Bottom
*Top vs Bottom is statistically different
p < 0.05
5 min
n=3
20 min
n=6
24 hrs
n=4
7 days
n=4
14 days*
n=4
21 days*
n=4
Time

Figure 2

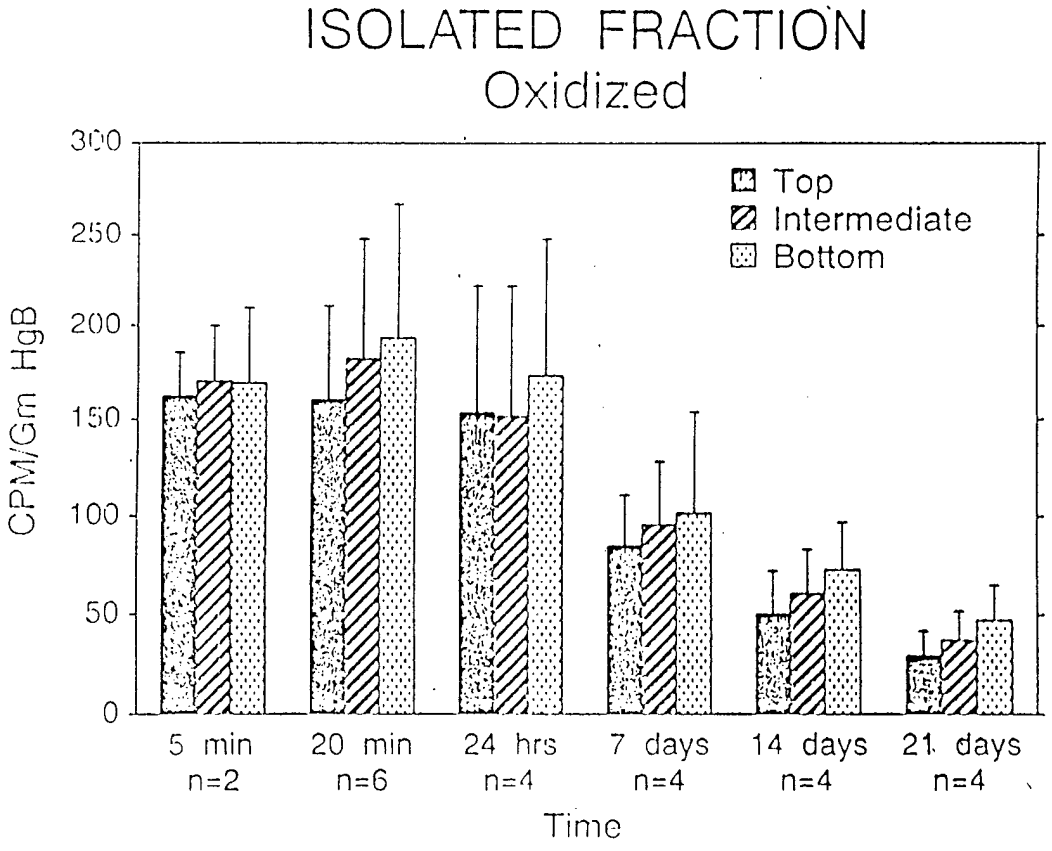
ISOLATED FRACTION
Oxidized
CPM/Gm HgB
300
250
200
150
100
50
0
Top
Intermediate
Bottom
5 min
n=2
20 min
n=6
24 hrs
n=4
7 days
n=4
14 days
n=4
21 days
n=4
Time

Figure 3

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